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Abstract

Dark fermentation using mixed cultures is a promising biotechnology for producing hydrogen (H_2) from renewable organic waste at a low cost. The impact of the characteristics of carbohydrates was evaluated on H_2 production and the associated changes in clostridial populations. A series of H_2-producing batch experiments was performed from mono-, di- to tri-saccharides (i.e. fructose, glucose, sucrose, maltose, cellobiose, maltotriose). Both chain length and alpha- or beta-linkage of carbohydrates impacted H_2 production performance as well as the patterns of hydrogenases. The H_2 yield, ranging from 1.38 to 1.84 mol-H_2/mol-hexose, decreased with the increasing chain length of the carbohydrates, showing a negative effect of the hydrolysis step on H_2 production efficiency. Changes in H_2 yield were associated with a specialization of clostridial species, which used different metabolic routes. The rise in H_2 production was associated with butyrate and acetate increases while H_2 consumption was related to caproate formation. Both clostridial [FeFe]- and [NiFe]-hydrogenases were identified in cellobiose cultures by a proteomic approach. This is the first study that combines genetic and proteomic analyses focused on H_2-producing bacteria under various conditions and it opens very interesting perspectives to better understand and optimize H_2 production using mixed cultures.

Keywords: hydrogen, *Clostridium*, CE-SSCP, *hydA* functional genes, anaerobic digestion, substrate

1. Introduction

Hydrogen (H_2) is one of the most promising, clean and renewable energy carriers. In recent years, considerable effort has been made to improve biological technologies for efficient, economic and renewable H_2 production [1, 2]. For large-scale H_2 production, dark fermentation processes using mixed cultures have some essential advantages over other bioprocesses: these include low energy requirements, high rates of production and considerable versatility of the organic substrates. Both purified sugar substrates and solid, waste-derived carbon sources, such as agricultural [3], food [4] or industrial waste [5], have been used to produce H_2. However, the main drawback of fermentative processes is the low yield of H_2 per mole of organic substrate consumed. To date, several strategies, such as metabolic pathway control or fermentation process optimization, have been proposed in the literature for improving H_2 production yields [1, 6, 7].

The H_2 production yield *via* dark fermentation varies widely according to the type of carbon substrate, the operating conditions and the type of H_2-producing bacteria (HPB) involved [8, 9]. Mixed cultures are potentially more suitable for degrading a wide range of complex and unsterile substrates. However, H_2 is a metabolic intermediate in anaerobic mixed cultures and is rapidly reused by H_2-consuming microorganisms (HCM). Therefore, in mixed cultures, the H_2 yields are not only influenced by the metabolic switches in bacterial cells but also by the population equilibrium between HPB and HCM [10]. Improvement in H_2 production yields using mixed cultures also relies heavily on microbial community dynamics which can respond to variations in operating conditions either by metabolic adaptation and/or by fluctuation in community composition. Moreover, HPB compete for selective carbohydrate uptake and metabolism to acquire a competitive advantage over other microorganisms [11, 12]. However,

only a few studies have reported changes in HPB population composition associated with changes in carbon sources [13, 14].

Previous studies of microbial communities have shown that HPB mixed cultures are usually dominated by clostridial species. This is due to heat treatment of the inoculum or the short hydraulic retention times often used to eliminate HCM (e.g. methanogens) [2, 15-18]. In fact, the clostridial species are recognized as effective H_2-producers using a wide range of carbohydrate substrates. Alterations in the functioning of ecosystem (such as a decrease in the H_2 yield in dark fermentation) are often associated with changes in the metabolism and composition of microbial communities [15, 16, 19, 20]. By way of example, shifts in microbial community from H_2-producing clostridial species to competitor HCM or non-H_2-producing bacteria such as lactic acid bacteria have been observed concomitantly with process instability. Such fluctuations can include H_2 production failure or variations in metabolite concentrations in the anaerobic bioreactors [21]. Some clostridial species are able to switch from H_2/acids to solvent production depending on the operating conditions [22, 23]. The metabolic flexibility of clostridial species leads to the production of a wide range of end products. Such variety may lower the H_2 yield from the theoretical maximum of 4 mol-H_2/mol-hexose which is obtained when acetate is the sole end product from fermentation. The genus *Clostridium* is a large and phenotypically heterogeneous bacterial group containing carbohydrate-fermenters, protein-fermenters and homoacetogens, all of which have not been phylogenetically well defined [24, 25]. H_2-producing and H_2-consuming clostridial species are in fact closely related phylogenetically, although they follow opposite physiological pathways [25, 26]. Consequently, changes in clostridial community make-up underlying changes in H_2 production performance can be difficult to observe using 16S rRNA gene-based methods [10, 16, 27].

H_2 production is catalyzed by the enzymatic activity of hydrogenases (H_2ases) which are classified in two major phylogenetically distinct classes according to their bimetallic center of the catalytic site: [NiFe]-H_2ases and [FeFe]-H_2ases [28]. While [NiFe]-H_2ases tend to be involved in H_2 consumption, [FeFe]-H_2ases are usually involved in H_2 production [28]. Indeed, in mixed cultures, the *hydA* genes, encoding the catalytic subunit of [FeFe]-H_2ases, have already been used as pertinent molecular markers of HPB in mixed cultures [16, 20, 29], in relation to changes in H_2 production performances [16, 29, 30]. Moreover, in relation to monitoring changes in H_2 production performances, the *hydA* population shifts have been reported as more sensitive than shifts in overall bacterial population [10, 20].

The aim of this study was to assess the impact of characteristics of carbohydrates on the bacterial population and on the metabolic changes associated with fermentative H_2 production. To this end, we evaluated the impact on H_2 production performances of carbohydrate chain length and alpha or beta linkages using mesophilic mixed cultures. The metabolic routes and the diversity of H_2ases used to produce or consume H_2 have also been discussed in the light of genetic and proteomic approaches.

2. Materials and methods

2.1. Hydrogen production in batch tests

The H_2 production experiments were carried out in 500 mL glass bottles under batch conditions. The inoculum corresponded to an anaerobically-digested sludge pretreated by heat/shock treatment (90°C, 10 min). Two milliliters of the pretreated inoculum (final concentration of 225 mg-COD/L) were inoculated into the culture medium (final working volume of 200 mL) containing 40 mM of 2-(N-morpholino)ethanesulfonic acid (MES) buffer and 10 g/L of the following carbohydrates: glucose ($C_6H_{12}O_6$, molar mass (M) = 180.2 g/mol, Sigma), fructose ($C_6H_{12}O_6$, M = 180.2 g/mol, Sigma), sucrose ($C_{12}H_{22}O_{11}$, M = 342.3 g/mol, Rectapur), maltose ($C_{12}H_{22}O_{11}$, M = 360.2 g/mol, Sigma), cellobiose ($C_{12}H_{22}O_{11}$, M = 342.3 g/mol, Sigma) or maltotriose ($C_{18}H_{32}O_{16}$, M = 504.4 g/mol, Sigma). The choice of the type of carbohydrate was based on the variety of potential substrates retrieved after hydrolysis of different renewable materials. Sucrose, a disaccharide consisting of one glucose monomer and one fructose monomer linked α-1,2, occurs naturally in various plant sources such as sugarcane or sugar beets. Maltose and cellobiose, both disaccharides consisting of two glucose monomers, the first linked α-1,4, the second linked β-1,4, are also both the products of the hydrolysis of polysaccharides: maltose comes from starch, cellobiose comes from celluolose. Maltotriose, a trisaccharide consisting of three glucose monomers linked α-1,4, is representative of linear glucose oligomers containing more than two units. The initial pH was adjusted to 5.5 using NaOH (1M). All batch tests were performed in triplicate. After inoculation, each bottle was flushed with nitrogen for 5 minutes to maintain anaerobic conditions. The bottles were then capped with a rubber stopper and incubated at 37°C for 14 days. Four milliliters of the mixed cultures were periodically collected and then centrifuged

(20,000 g, 10 min). The supernatants and the pellets were stored at -20°C, the supernatants for further chemical analysis and the pellets for DNA extraction.

2.2. Chemical data analysis (biogas, metabolic byproducts)

The volume of biogas was daily measured using an acidified water displacement method. Biogas composition (CH_4, CO_2, H_2 and N_2) was determined by gas chromatography (GC-14A, Shimadzu) as previously described by Aceves-Lara *et al.* [7].

Volatile fatty acids (VFA) composition in the liquid phase, *i.e.* acetic (C2), propionic (C3), butyric and iso-butyric (C4 and iC4), valeric and iso-valeric (C5 and iC5) and caproic (C6) acids were determined with a gas chromatograph (GC-3900, Varian) equipped with a flame ionization detector. The concentrations of carbohydrates and non-VFAs metabolic byproducts such as organic acids (lactate), ethanol or acetone were assessed by HPLC analysis and a refractometer (Waters R410). The compounds were separated using an Aminex HPX-87H, 300 x 7.8 mm column (Biorad). The column temperature was maintained at 35°C and the flow rate at 0.4 mL/min. Substrate degradation efficiency was estimated by dividing the amount of carbohydrate consumed by the amount of initial carbohydrate.

To determine the H_2 production performances obtained for the different carbohydrates (Table 1), the cumulative H_2 production (H) data for each carbohydrate was fitted to a modified Gompertz equation (1) as shown in Figure 1.

$$H(t) = P.\exp\left\{-exp\left[\frac{R_m.\mathrm{e}}{P}(\lambda - \mathrm{t}) + 1\right]\right\} \quad (1)$$

where P is the H_2 production potential (mL/L), R_m is the maximum H_2 production rate (mL/L/day), λ is lag-phase time (day), t is the incubation time (day) and e is exp(1). The cumulative H_2 production was expressed in mL per L of culture taking into account the volume variations due to gas and liquid samplings. The values of P, R_m and λ were estimated

using a non-linear regression algorithm developed in Matlab (version 6.5, Mathworks). The H_2 consumption rate (R_c) was evaluated by the H_2 decrease measured in experiments between the P time and the end of the experiments. The H_2 production yield was calculated by dividing the maximum H_2 production potential (*P*) by the mol of hexose-equivalent consumed.

2.3. DNA extraction and PCR amplification

For each experiment, molecular analyses of the bacterial communities were carried out in triplicate at the start of the experiments (initial time), at the H_2 production potential stage (*P*) and at the end of the experiments (after 14-day incubation).

Genomic DNA was extracted and purified from cell pellets using the Wizard Genomic DNA Purification kit (Promega). The amount and purity of DNA in the extracts were measured by spectrophotometry (Infinite NanoQuant M200, Tecan).

The *hydA* genes were amplified using the non-degenerated primers [16] *hydAClos*F (5' – ACCGGTGGAGTTATGGAAGC – 3', *C. pasteurianum* position F1258) and 5′-fluorescein phosphoramidite-labeled *hydAClos*R (5' – CATCCACCTGGACATGCCAT – 3', *C. pasteurianum* position R1508). Each PCR mixture (50 µL) contained 1X *Pfu* Turbo DNA polymerase buffer, 200 µM of each dNTP, 0.5 µM of each primer, 0.5 U of *Pfu* Turbo DNA polymerase (Stratagene) and 1 ng of genomic DNA. Reactions were done in a Mastercycler thermal cycler (Eppendorf). The *hydA* genes were amplified as follows: 94 °C for 2 min, followed by 35 cycles performed at 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 10 min.

The 16S rRNA genes were amplified using the universal primers W49 (5′-ACGGTCCAGACTCCTACGGG-3′, *Escherichia coli* position F331) [31] and 5′-fluorescein phosphoramidite-labeled W104 (5′-TTACCGCGGCTGCTGGCAC-3′, *E. coli* position R533)

[31], as described above, except that 25 cycles and primer hybridization at 61 °C were applied and 130 ng of each primer were used. The *Clostridium* genus was specifically analyzed by nested PCR using a bacterial-domain forward primer W18 (5′-GAGTTTGATCMTGGCTCAG -3′, *E. coli* position F9) [32] and a *Clostridium*-specific reverse primer W109 (5′- CCCTTTACACCCAGTAA -3′, *E. coli* position R561) [33, 34]. The size of PCR products (200 bp) was determined by 2% gel electrophoresis stained with ethidium bromide.

2.4. CE-SSCP electrophoresis and statistical analysis

Electrophoresis- One microliter of appropriate dilution of PCR product was mixed with 18.8 µL of formamide and 0.2 µL of internal standard GeneScan ROX (Applied Biosystems). Samples were heat-denatured at 95°C for 5 min and immediately cooled in ice. CE-SSCP electrophoresis was performed in an ABI Prism 3130 genetic analyser (Applied Biosystems) with 50 cm-long capillary tubes filled with a non-denaturing 5.6% conformation analysis polymer (Applied Biosystems). Samples were eluted at 12 kV and 32°C for 30 min (for 16S rRNA gene) or 45 min (for *hydA* gene).

Data analysis- The CE-SSCP profiles were aligned with the internal standard to take into account inter-sample electrophoretic variability. The CE-SSCP profiles were normalized using the StatFingerprints library [35] from R version 2.9.2 [36] in accordance with standard procedure [37]. The genetic distances between bacterial communities were assessed using Euclidean distances. The effect of the type of carbohydrate on the structure of bacterial communities was investigated with a multivariate analysis of similarity (ANOSIM) [38]. ANOSIM gives a statistic, R, ranging from 0 (dissimilar groups) to 1 (similar groups). The statistical significance of R was tested by Monte Carlo randomization. The complexity of the

bacterial community was estimated using Simpson's diversity index from a CE-SSCP profile taking into account the number of species (number of peaks) as well as their relative abundance (area under each peak) [39].

2.5. Assignation of the partial *hydA* sequences in mixed cultures

The PCR products obtained from the H_2-producing mixed cultures were purified using a PCR Purification Kit (Qiagen). The *hydA* clone libraries were constructed using the TA Cloning Kit (Invitrogen). The PCR-CE-SSCP profile of each PCR product, amplified using plasmid-targeted primers T7 and P13, was compared with the HPB culture profiles for peak assignation. The cloned inserts were sent for sequencing (MilleGen Company, Toulouse, France). The protein sequences, deduced from the nucleotide *hydA* sequences, were aligned with reference sequences retrieved from the Genbank database using the CLUSTALW program [40] then further refined manually using the BioEdit program [41]. Distances were estimated by the Kimura method [42] and the neighbor-joining method was used for inferring the tree topologies [43]. Tree topology confidence was determined by bootstrap analysis on 1000 replicates [44]. All phylogenetic programs were implemented with SeaView software [45]. The *hydA* gene sequences were deposited in the Genbank database under the accession numbers JF714976 to JF714980.

2.6. Protein electrophoresis and proteomic data analysis

Electrophoresis- Cell material was collected at the end of the experiment (14 days). Soluble proteins released from dead bacterial cells were eliminated prior to protein extraction by rinsing cell pellets. Washed pellets were resuspended in 50mM Tris-HCl, 10 µg/ml Dnase,

5% glycerol and proteases inhibitors (pH 7.6) (bufferA), mutanolysin (200U/ml) (Roche) and incubated 1hour at 37°C under argon. After centrifugation (30 min 3700g, 4°C), cell material was resuspended in buffer A and broken in a French cell press. Debris and unbroken cells were removed by centrifugation (10000g, 15 min). Membrane and soluble proteins were separated by ultracentrifugation (45 min, 40000 rpm, rotor 45 Ti, Beckman). Proteins were separated on denaturing gels [46]. A mini-Protean III cell electrophoresis apparatus (Bio-Rad) was used in all cases. After migration, gels were either stained with Coomassie Blue R-250, enzymatic activities were revealed and proteins were detected with anti-H_2ase antibodies. Protein bands were cut out from gels and stored at -20°C prior to mass spectrometry analysis.

Western Blotting- Proteins were subjected to SDS gels and transferred to nitrocellulose membranes (Electran) with the Fast blot apparatus (Biometra) at 5 mA/cm^2 for 30 min. The blots were then processed and developed as previously described by Castelle et al. [47]. Primary antibodies used were directed against H_2ase from *A. aeolicus* or *Desulfovibrio*.

Tryptic digestion of excised gel plugs, Ion Trap and MALDI-TOF mass spectrometry protein identification were performed, as previously described by Guiral et al. [46].

Data analysis- Protein identification was done by the TurboSEQUEST algorithm (version 28, rev. 12) using the NCBI database (02/28/2006, http://www.ncbi.nlm.nih.gov) restricted to the *Clostridium* genus containing 498879 entries. The spectra were analyzed for searching for average peptide peaks (m/z 600 to 3500 Da). Search parameter criteria were set as follows: two missed cleavage sites accepted variable methionine oxidation, cysteine carbamidomethylation, 1.5 and 1.0 Da as maximum precursor and fragment tolerance. Protein identification was considered to have been obtained when at least 2 unique peptides in 2 independent experiments were detected. In our analyses, some peptides matched with different H_2ases from various strains. Only the strains displaying various identified proteins were accepted.

3. Results

3.1. Effect of carbohydrate type on overall hydrogen production performances

During the course of the experiments, only H_2 and CO_2 were produced as gaseous products, with no detectable CH_4, indicating that the heat pretreatment was effective in suppressing methanogen activity. The Gompertz model provided a good fit for the H_2 production data, with determination coefficients R^2 over 0.98 for all batch tests (Table 1).

The type of substrate had a significant effect on H_2 production performance (Table 1). Among different kinds of carbohydrates, fructose and glucose cultures gave the best H_2 production potentials (*P*) (2.31 and 2.24 L/L, respectively). These monosaccharide cultures were faster in reaching their peak of H_2 production than other disaccharide and trisaccharide cultures (7.7 and 9.7 days, respectively). The highest yield (1.84 mol-H_2/mol-hexose) was obtained with fructose and the lowest with maltotriose (1.38 mol-H_2/mol-hexose), showing that the higher the substrate chain length, the lower the H_2 production yield. Indeed, a significant negative correlation was observed between the H_2 yield and the number of monomer units (Pearson's correlation coefficient $r = -0.82$, $p < 0.005$), indicating a negative impact from the hydrolysis step. Among disaccharide cultures, higher H_2 yields were observed from alpha-linked maltose cultures than beta-linked cellobiose cultures, suggesting an effect of the glycoside linkage type.

Whatever the substrate, the H_2 production phase was followed by a H_2 consumption phase (Figure 1). Indeed, the H_2 content in the gas phase increased until reaching between 31.0% and 34.2% at the *P* time; it then decreased to 22.7%-27.6% at the end of the experiments. The highest H_2 consumption rate (R_c) was observed in the cellobiose cultures (Table1).

As expected, the pH decreased over time from 5.5 to 4.0 in average. The pH drop was faster for the most efficient monosaccharides cultures when compared to di- and tri-saccharides. A significant negative correlation was observed between pH and H_2 production (Pearson's $r > -0.97$, $p < 0.001$). However, no significant pH difference was observed using different carbohydrates at *P* time despite the difference in H_2 production and yield (final pH = 4.0 ± 0.0) (Table 1). Moreover, no significant change in pH was observed during the H_2 consumption phase, despite the change in metabolic patterns (Table 2).

3.2. Effect of carbohydrate type on the fermentative routes

Different distributions of the metabolic products were observed depending on the substrate, although acetic and butyric acids were the major VFAs produced in all batch tests (Table 2). Indeed, after *P* was reached the acetate and butyrate molar content within the soluble microbial products ranged from 24.7% to 30.4% and from 58.0% to 69.7%, respectively. This result indicates that acetate-butyrate fermentation occurred whatever the substrate used. The highest acetate and butyrate levels were observed in sucrose cultures, where high *P* was obtained. The other byproducts corresponded to caproate, ethanol and lactate (1.5 - 2.7 %, 2.8 - 10.5 % and 0 - 3.9%, respectively). Similar metabolic product distributions were observed in the monosaccharide cultures. In contrast, significant differences in metabolic profiles were obtained with the three different disaccharides: sucrose, cellobiose and maltose. The highest caproate, ethanol and lactate contents were obtained in the cellobiose cultures, a result which corroborates the negative influence of the beta-linkage type on H_2 production. When *P* was achieved, caproate production was only detected in the cellobiose-, maltose- and maltotriose cultures, where lower H_2 yields were obtained (Table 1). The least effective maltotriose culture was characterized by the highest concentrations of caproate, ethanol and lactate at *P* time (Table 2). Interestingly, and in total contrast to the maltotriose culture, the maltose

culture showed the lowest caproate, ethanol and lactate levels, indicating the effect of substrate chain length on the fermentation route. No correlation was observed between H_2 production performance and the ratio of butyrate to acetate concentrations (Bu/Ac) (Table 2) when *P* was reached (Pearson's $r = 0.35$, $p > 0.05$). At the final stage, a decrease in the Bu/Ac ratio was observed in all batch tests during the H_2 consumption phase (Table 1). Except for fructose culture, a significant decrease in butyrate concentrations was observed after 14-day incubation. Concomitantly, the caproate end product accumulated significantly in the disaccharide cultures, indicating that different metabolic pathways were being utilized at the end of the experiment.

3.3. Effect of carbohydrate type on the hydrogen-producing bacterial populations

Similar bacterial- and *Clostridium*-specific 16S rDNA CE-SSCP profiles were obtained during the 14 days of incubation, supporting the conclusion that clostridial species enriched by heat-shock pretreatment dominated the H_2-producing mixed cultures (data not shown). In Figure 2, the *hydA* gene-based CE-SSCP profiles are presented for the *P* point reached. No significant change in the HPB population was observed between the *P* time and the end of the experiments despite the presence of the H_2-consumption phase. The phylogenetic affiliation of the eight different *hydA* genes, the molecular markers of HPB, is illustrated in Figure 3. The retrieved sequences were related to various species among the *Clostridium* genus: *Clostridium acetobutylicum*, *Clostridium cellulolyticum*, *Clostridium kluyveri*, *Clostridium saccharobutylicum* and *Clostridium sporogenes*.

Overall, the type of carbohydrate had a significant effect on the HPB population structures (ANOSIM $R = 0.85$, $p < 0.005$). The fructose, glucose or sucrose cultures, which showed similar *P* values and fermentation end-products, displayed similar *hydA* CE-SSCP profiles (Figure 2). Peak 1 (70% identity with the HydA sequence of *C. sporogenes*) dominated the

CE-SSCP profiles of the glucose, fructose, sucrose and the cellobiose cultures. The HydA sequences corresponding to CE-SSCP peaks 2, 4 and 5 detected in the sucrose- and cellobiose cultures were related to that of *C. acetobutylicum* (86-88.6% identity). The cellobiose cultures displayed the highest Simpson's diversity index values (2.8 ± 0.3) compared to other cultures (ranging from 1.4 to 1.9). The HydA sequence from peak 3 was related to *C. cellulolyticum* (72.5% identity) which belongs to the *Clostridium* cluster III. The HydA sequence from peaks 6 and 7 were affiliated, respectively, to those of *C. saccharobutylicum* (95.2% identity) and *C. kluyveri* (79.4% identity). Finally, the CE-SSCP profiles of maltose and maltotriose cultures differed from those of other carbohydrate cultures by the predominance of another HydA sequence corresponding to peak 8 and related to *C. acetobutylicum* (87.5% identity).

3.4. Hydrogenase identification by metaproteomic approach

No substantial difference in the total protein pattern was observed between the various substrate cultures at the end of the experiments, assumed as the end of the H_2 consumption phase. An example of total protein pattern is presented in Figure 4A. Various bands were labeled by the antibodies which confirmed the presence of various H_2ases in the samples (Figure 4B). The major difference was observed between the cultures on cellobiose and those on the other substrates (e.g. glucose and fructose), in accordance with CE-SSCP data. The H_2ases present in the fraction were precisely identified by proteomic analysis (Table 3). The H_2ase-like proteins were related to those from the different species of the *Clostridium* genus: *Clostridium carboxidivorans*, *C. cellulolyticum*, *Clostridium novyi* and *C. sporogenes*. The proteins identified from glucose and fructose cultures were [FeFe]-H_2ases and a subunit homologous to the [2Fe-2S] subunit from NADH dehydrogenase. In the cellobiose samples, [FeFe]-H_2ase and the Ech H_2ase (i.e. *E. coli* H_2ase 3-type) subunit homologous to [NiFe]-

352 H_2ase were identified (Table 3). From the membrane fraction of the cellobiose cultures,
353 subunit membrane-bound complexes were identified as belonging to the Ech H_2ase group,
354 proposed as H_2-producing [NiFe]-H_2ases ([NiFe]-H_2ase group 4, as defined by Vignais et al.
355 [28, 48]).
356

4. Discussion

In this paper, a gradually increasing H_2 yield was observed from mesophilic clostridial mixed cultures depending on the chain length: fructose and glucose (monosaccharides) > sucrose, maltose and cellobiose (disaccharides) > maltotriose (trisaccharide). These results demonstrate that glycosidic hydrolysis was less adapted to efficient H_2 production. These findings are consistent with those obtained from mesophilic clostridial consortia isolated from riverbed sediments using different sugar sources [14]. In contrast, Ferchichi et al. [49] found that disaccharides (lactose, sucrose and maltose) produced twice as much H_2 as monosaccharides (glucose and fructose) using a pure culture of *Clostridium saccharoperbutylacetonicum* ATCC 27021. In addition, Yokoyama et al. [13] achieved similar H_2 yields (2.68-2.65 mol-H_2/mol-hexose) from thermophilic anaerobic microflora using cellobiose and glucose as the substrate. The optimal type of substrate for H_2 production by our mesophilic mixed culture varied no doubt because of a different selection of clostridial HPB species. In this study, it is noteworthy that an effect of the glycoside linkage on H_2 production was shown by comparing cellobiose and maltose culture performances. A similar glycoside linkage effect with opposite results has already been reported in a hyperthermophilic pure culture of the archaeon *Pyrococcus furiosus* [50]. Chou et al. [50] proposed that different H_2 productions from cellobiose and maltose by *P. furiosis* were due to changes in metabolic routes. In our mixed cultures, the different H_2 yields can be explained by changes in both HPB population and metabolic routes.

To date, glucose has been the most commonly-used carbon source for determining H_2 production ability in laboratories. The H_2 yields obtained from our glucose cultures (1.79 mol-H_2/mol-hexose) were slightly higher than the reported yields of 1.46 mol-H_2/mol-glucose

382 [51] and 1.67 mol-H_2/mol-glucose [52] from comparable batch tests using mixed microbial
383 cultures of heat-pretreated anaerobic sludge. In contrast, clostridial pure cultures, such as *C.*
384 *acetobutylicum* [53] and *C. saccharoperbutylacetonicum* ATCC 13564 [54] exhibited higher
385 H_2 yields (2.0 and 3.1 mol-H_2/mol-glucose, respectively) than our clostridial mixed cultures.
386 Using cellobiose as substrate, Ren et al. [55] also achieved higher H_2 yields in different pure
387 cultures (e.g. *C. acetobutylicum* and *C. cellulolyticum*) compared to the yield in our mixed
388 cultures: their results ranged from 1.6 to 2.3 mol-H_2/mol-hexose, ours was 1.5 mol-H_2/mol-
389 hexose. Interestingly, *C. acetobutylicum* and *C. cellulolyticum* were detected in our cellobiose
390 cultures (Figure 2 and 3). These results confirm that in general mixed cultures produce less H_2
391 than pure cultures with pure substrates. These results can be explained first of all by the
392 operating conditions, which were probably not optimal for the growth of such recognized
393 efficient HPB as *C. acetobutylicum*, *C. cellulolyticum* or *C. sporogenes*. An example here is
394 the initial pH of our medium which was adjusted to 5.5. This is lower that the optimal pH of
395 *Clostridium* species, even though this pH is known as optimal for H_2 production in mixed
396 cultures. But the low H_2 yield from mixed cultures can be related to the presence of clostridial
397 HCB and negative inter-species interaction occurring in complex anaerobic microbial
398 communities. Such biotic interactions have already been observed in artificially-engineered
399 fermentative ecosystems used in cheese ripening [56].
400
401 The variations in H_2 production performance were associated with changes in metabolic
402 profiles and HPB populations. The more efficient HPB cultures dominated by *C. sporogenes*
403 were characterized by high levels of acetate and butyrate production, each with pathways
404 linked to H_2 production; and, simultaneously, by low ethanol and lactate yields, as alternative
405 but non-H_2-producing routes. Indeed, clostridial species are usually known to produce H_2
406 from a wide variety of carbohydrates *via* the butyrate-acetate fermentation route [2]. More

407 precisely, *C. sporogenes* is known to ferment mainly amino acids [57]. Recently, the
408 consumption of H_2 produced by glucose- or starch- fed HPB cultures amended by peptone has
409 been associated with the presence of *C. sporogenes* [58]. In glucose or maltotriose cultures,
410 H_2 consumption was concomitant with an increase in acetate. Consequently, H_2 consumption
411 detected in our cultures was most probably due to homoacetogenesis: it is a favored reaction
412 at pH 5.5 producing only acetate from H_2 and CO_2 [59]. In the disaccharide cultures, H_2
413 consumption probably also resulted from caproate formation [10, 60]. Indeed, the increase in
414 caproate concentration between the *P* and the final stages was associated with decreases in
415 concentrations of butyrate, CO_2 and H_2. These findings are in agreement with another
416 pathway for caproate formation using sucrose as the substrate in fermentative HPB mixed
417 cultures. These cultures consumed equimolar butyrate, H_2 and CO_2 in a thermodynamically-
418 favorable reaction (ΔG= -143.34 kJ) [60]. Interestingly, previous studies on *C. kluyveri* during
419 H_2-producing glucose fermentation have reported the possibility of caproate formation from
420 ethanol and acetate or ethanol and butyrate [61].
421
422 The changes in HPB population structure associated with variations in the H_2 yield as a
423 function of the different carbohydrates was particularly interesting because each pure
424 clostridial culture was reported as metabolizing each of the six substrates tested [62].
425 Although glucose is the preferred carbohydrate of many clostridial species, such species have
426 metabolic resources flexible enough to ferment a wide range of carbohydrates [12]. For
427 instance, *C. acetobutylicum* can metabolize glucose, cellobiose and maltose [55, 62]. Thus the
428 variation in H_2 yields may be due entirely to the use of different mechanisms for the
429 hydrolysis of the glycoside linkage and transport [12]. In this study, however, the structure of
430 the HPB populations changed depending on the type of the glycoside linkage and the chain
431 length. A single dominant HPB, i.e. *C. sporogenes* was selected in glucose, fructose and

sucrose cultures, cultures which presented the highest H_2 yield and similar metabolic profiles. It is noteworthy that *C. sporogenes* has been reported previously as the dominant, key HPB species in mesophilic mixed cultures using glucose or sucrose as the substrate [16, 58, 63]. However, different HPB populations established on cellobiose, maltose and maltotriose have been associated with different metabolic profiles. Remarkably, a more diverse HPB population made up of *C. sporogenes, C. cellulolyticum, C. acetobutylicum, C. saccharobutylicum* and *C. kluyveri* emerged with cellobiose as the substrate. The use of beta-linked carbohydrates for H_2 production by mesophilic mixed cultures seems to require a wider range of clostridial species. These results suggest that cooperation between HPB can be obtained on beta-linked carbohydrates whereas competition seems to occur on unlinked and alpha-linked carbohydrates. *C. acetobutylicum* and C. *cellulolyticum* have been previously reported as efficient H_2 producers on cellobiose [55, 64]. Like other clostridial species, *C. saccharobutylicum* is able to grow on a wide range of carbohydrates, including cellobiose [62]. *C. kluyveri* can grow anaerobically with ethanol and acetate as its sole source of energy [65]. Interestingly, extreme thermophilic anaerobic microflora displayed less diversity of 16S rRNA genes when enriched by cellobiose rather than glucose [13]. Yokoyama et al. [13] enriched fairly similar microbial communities whatever the type of hexose while a clearly different microbial community was enriched on xylose. Significant difference between microbial communities grown on glucose or xylose have also been observed previously in fermentative mixed cultures [19]. In our study, changes in dominant HPB species were observed only when using different hexose-based carbohydrates. Overall, these findings indicate firstly that clostridial HPB structure is sensitive to substrate change and secondly that a specialization of the clostridial population occurs depending on the characteristics of the carbohydrate used with mixed cultures. Shifts in the dominant HPB species associated with variations in the H_2 yield have already been observed in batch experiments run at different

temperatures [16]. Moreover, as observed in cellobiose cultures, the increase in *hydA* gene diversity was also detected in previous experiments with higher pH [10]. In this study, changes in both diversity and the dominant HPB were observed depending on the type of carbohydrate.

Using the metaproteomic approach, various proteins associated with clostridial species were identified from mixed cultures. From glucose or fructose cultures at least two different [FeFe]-H_2ases with different molecular mass were detected in the soluble fraction. Recently, Calusinska et al. [66], on the basis of genome analysis, pointed out that some clostridial species contain numerous distinct [FeFe]-H_2ases presumably to allow them to adapt quickly to changing conditions. The species identified in glucose and fructose substrates are linked to [FeFe]-H_2ases from *C. novyi*, *C. carboxidivorans* and *C. sprorogenes*. These clostridial species present various [FeFe]-H_2ases belonging to the group A6, B2 and B3 [66]. Using the metaproteomic approach, [FeFe]-H_2ases similar to those belonging to B2 and B3 types were clearly identified. A protein belonging to the NADH dehydrogenase family was also identified in the soluble fraction (Figure 4B, band d). This protein is similar to [2Fe2S] ferredoxin. Analysis of the genome context showed that the corresponding genes belonged to, respectively, an operon encoding the [FeFe]-H_2ase and the NADH quinone dehydrogenase. The NADH subunit identified belonged to the group A8. This latter group seemed to be NAD-dependent and was proposed as "heterodimeric bifurcative" H_2ase [66]. However as the other subunits were not identified, we cannot affirm that this enzyme was present in our conditions. Using cellobiose as the substrate, only one [FeFe]-H_2ase was detected similar to that from *C. cellulolyticum* (group A1). A second H_2ase was identified in both the soluble as well membrane fractions. This subunit belongs to the [NiFe]-H_2ase family. Analysis of the genome showed that it was located in an operon with at least three other genes similar to Ech

H_2ases. This enzyme is similar to the [NiFe]-H_2ases belonging to the group 4 defined by Vignais et al. [28, 48] and was closely related to the NADH:ubiquinone oxidoreductase. This enzyme has been identified in various *Bacteria* and *Archaea* and its presence in *Clostridia* has been the subject of speculation on the basis of genome analysis only: there is no data concerning the function of this enzyme in *Clostridia*. In methanogens, it plays a role in the first stage of methanogenesis. In *Desulfovibrionaceae* family, Ech H_2ase can work bidirectionally, depending on the growth conditions [67]. This amounts to the first identification of this group of H_2ase in *Clostridia*. [NiFe]-H_2ases belonging to group 1 (defined by Vignais et al. [28, 48]) was not detected in this study. These [NiFe]-H_2ases are usually involved in H_2 uptake linked with energy metabolism and to our knowledge have only been detected in the clostridial genome by sequence homology. Their physiological roles as well as their protein properties in clostridial species are unknown.

Both the genetic and proteomic analyses highlighted changes in H_2ases diversity associated with changes in carbohydrate type and, thus, H_2 production performances. Changes in genetic diversity in [FeFe]-H_2ases have been previously demonstrated in relation to H_2 production performances [15, 20]. Proteomic shifts have also been associated with metabolic shifts during fermentation carried out by clostridial species [22, 23]. [FeFe]-H_2ases sequences from similar clostridial species were recovered from the H_2-producing mixed cultures by these two different molecular approaches. Like other primers currently cited in the literature and targeting the cluster H of the *hydA* genes, the primers used in this study were only able to target the sequences of Group A of [FeFe]-H_2ases, as defined by Calusinska et al. [66]. Consequently, the [FeFe]-H_2ases belonging to group B, which were highlighted by the proteomic approach and thus assumed to be active in the cultures, could not be detected by the genetic fingerprinting method. Even so, it is important to emphasize that the *hydA* CE-

SSCP method has been proven helpful in providing an overview of HPB diversity and structure in numerous samples from dark fermentation bioreactors. It is also useful in comparing variations in such diversity and structure in response to changes in environmental parameters [10, 16]. Using proteomic techniques, [Ni-Fe]-H_2ases were detected in cellobiose cultures, cultures which showed the highest H_2 consumption rates. Recently, plasmid or chromosomal genes encoding [NiFe]-H_2ases have been detected in the genome of some members of the *Clostridium* genus such as *C. acetobutylicum*, *C. cellulolyticum* or *C. sporogenes* [66]. Interestingly, as found in this study, these clostridial strains were detected during the H_2 consumption phase of previous batch experiments carried out in our laboratory [10]. Contrary to [FeFe]-H_2ases and [NiFe]-H_2ases from *Proteobacteria*, [NiFe]-H_2ases from *Clostridia* have not yet been studied and their physiological functions are still unknown [66]. Consequently, it is still unclear whether clostridial [NiFe]-H_2ases can be associated with H_2 consumption in dark fermentation bioreactors. Further research is needed to determine the physiological roles played by the different [Fe-Fe]- and [Ni-Fe]-H_2ases in clostridial species. To further analyze the role of these enzymes, differential transcriptome analyses of H_2ase-encoding genes under different operating conditions could well provide the basis for future work.

5. Conclusions

The characteristics of carbohydrates were found to be important criteria influencing the performance of dark fermentation systems. The hydrolysis of carbohydrate was shown as a critical step in the H_2 production pathway. Monosaccharides led to more efficient H_2 production performances when compared to di- and tri-saccharides. Moreover, the negative effect of the glycoside chain length was amplified by the glycoside beta-linkage type. The difference in performance with various carbohydrates was related to the enrichment of different specialized clostridial species, which used diverse metabolic routes. Finally, we have expanded the diversity spectrum of clostridial H_2ases potentially involved in H_2 metabolism in fermentative mixed cultures by using a combination of proteomic and molecular genetic approaches. Future work will undertake to continue clarifying the function of these diverse H_2ases in clostridial species and to identify the key regulatory pathways in engineered/natural H_2-producing ecosystems.

Acknowledgements

We gratefully acknowledge the financial support from the 'Institut National de la Recherche Agronomique' (INRA) through a postdoctoral fellowship. This work was funded by the InGEcoH Project from the French National Research Agency (ANR) (ANR contract number 2008-BIOE-005-01). We also gratefully acknowledge the contribution of Pacale Infossi (BIP) for technical assistance and Sabrina Lignon and Régine Lebrun (Proteomic Analysis Center, IFR88, CNRS, Marseille) for proteomic analysis. BIP was financially supported by the CNRS, the Région-Provence-Alpes Côte d'Azur and Marseille Metropole. We thank Tim Sparham for revising the English of the manuscript. We would like to thank the reviewers for her/his comments that help us to improve the quality of our paper.

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746

Captions to figures

Figure 1. Representative dynamics of H_2 production from a mixed culture cultivated with fructose as substrate and heat-treated anaerobic digested sludge as inoculum. Experimental data (triangles) and predicted Gompertz equation (solid lines) are shown. The estimated values of *P*, R_m and λ correspond to the H_2 production potential (mL/L), the maximum H_2 production rate (mL/L/day) and the lag-phase time (day), respectively. The R_c value, estimated by the H_2 decrease experimentally measured between the *P* time and the end of the experiments, corresponds to the H_2 consumption rate.

Figure 2. CE-SSCP profiles of *hydA* gene fragments retrieved from H_2-producing experiments conducted from different carbohydrates, at maximum H_2 production yields. The different CE-SSCP profiles were aligned on the basis of the common ROX internal standard, and areas normalized. The x and y axis of each CE-SSCP profile represent, respectively, the relative peak migration distance and the relative peak intensity. A representative CE-SSCP profile from the three replicates is presented for each substrate. The dominant peaks of each CE-SSCP profile are marked with a number. The phylogenetic affiliation of the sequenced clones that match a numbered peak is given in Figure 3.

Figure 3. Neighbor-joining phylogenetic tree of the HydA sequences (134 amino acids). The Genbank accession numbers (in brackets) were obtained from the protein sequence databases. The sequences obtained in this study are in bold and highlighted. Bootstrap values (n = 1000 replicates) are indicated as grey circles (<50%). The scale bar indicates 5% sequence divergence. The *Desulfovibrio* HydA sequences (AAS96246, AAA23373 and CAA72423) were used as outgroups.

772

773 **Figure 4.** (A) SDS polyacrylamide gel of soluble crude extract. Lane 1: Molecular mass
774 markers (in kDa). Lane 2: 200 µg of crude extract. (B) Immunoblotting experiments of crude
775 extract from cellobiose (Lane 1) fructose (Lane 2), glucose (Lane 3), maltose (Lane 4) and
776 sucrose (Lane 5) demonstrating the presence of hydrogenases. Molecular mass markers were
777 given in kDa.

Figure1

Figure 1

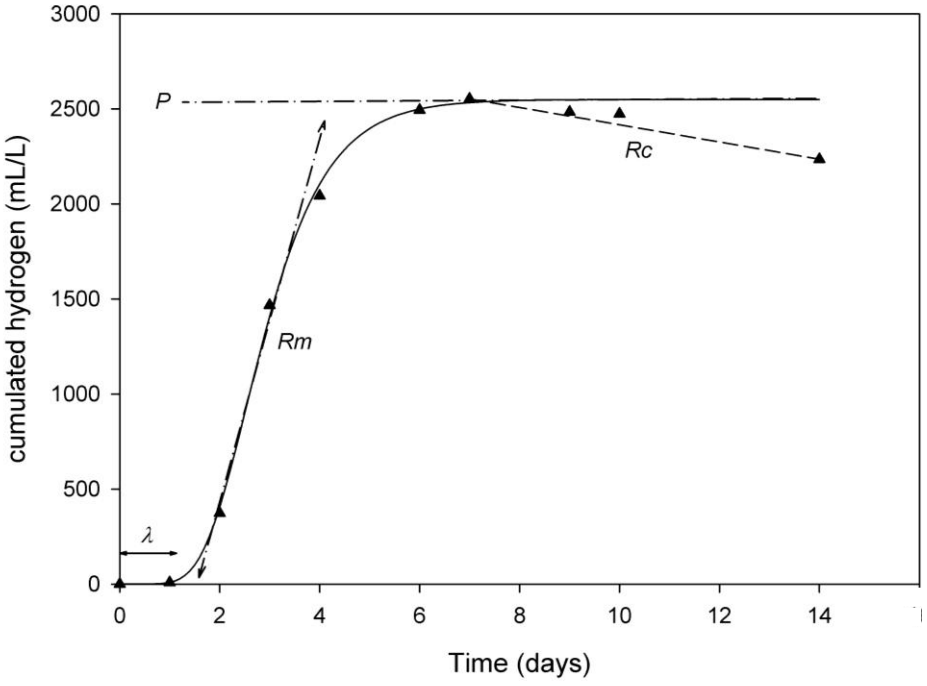

Figure2

Figure 2

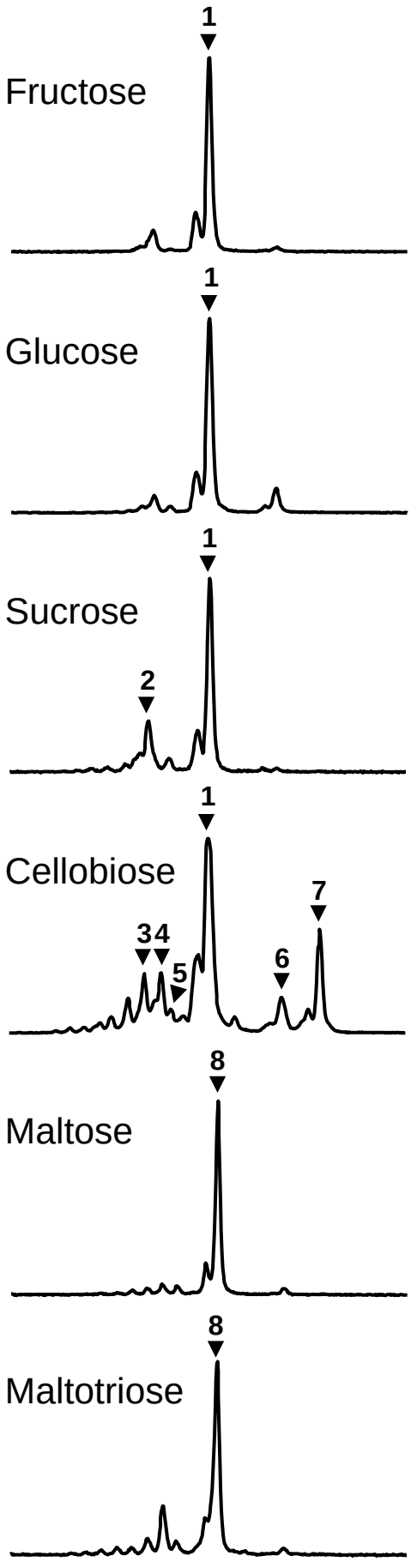

Figure3

Figure 3

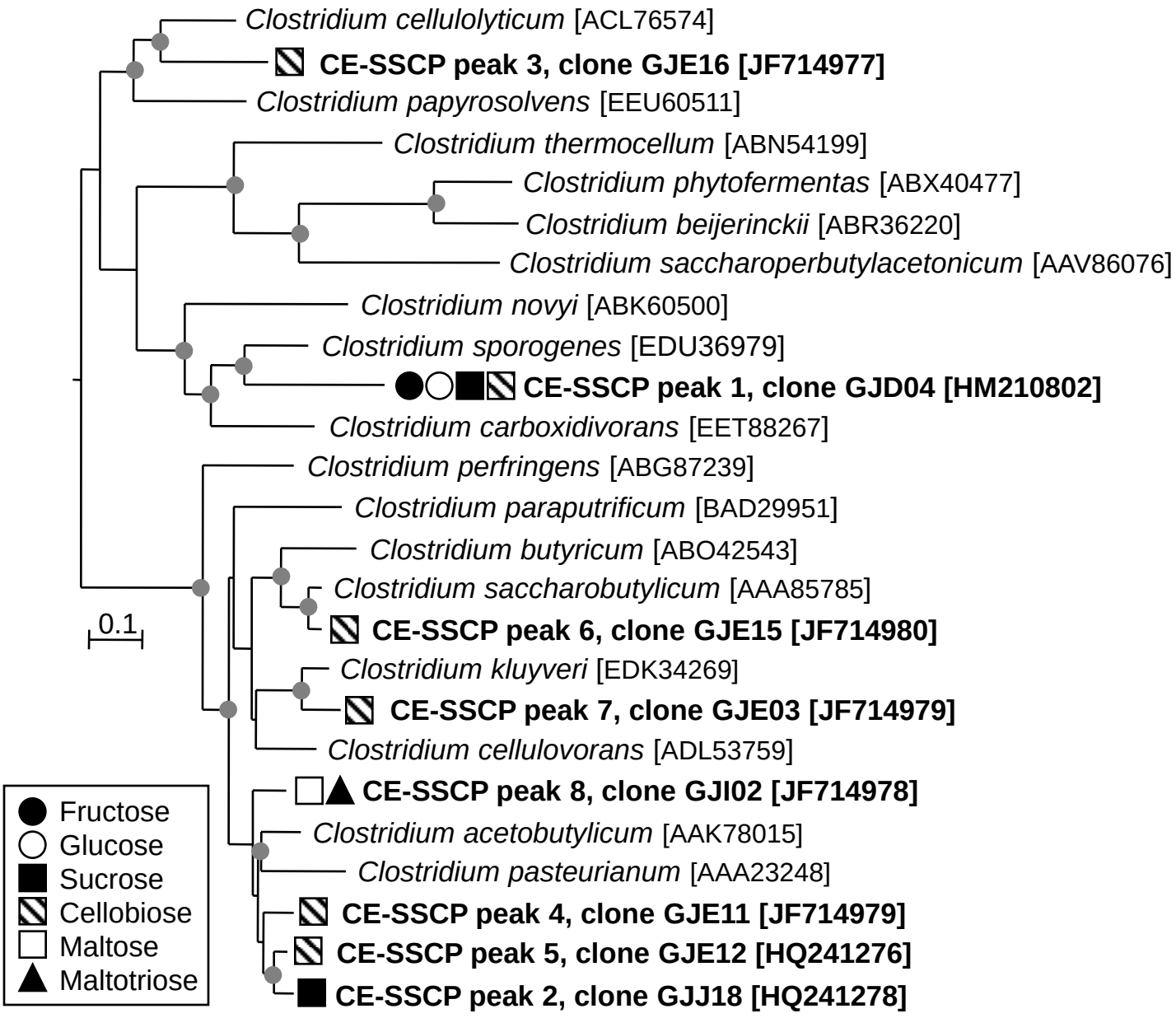

Figure4

Figure 4

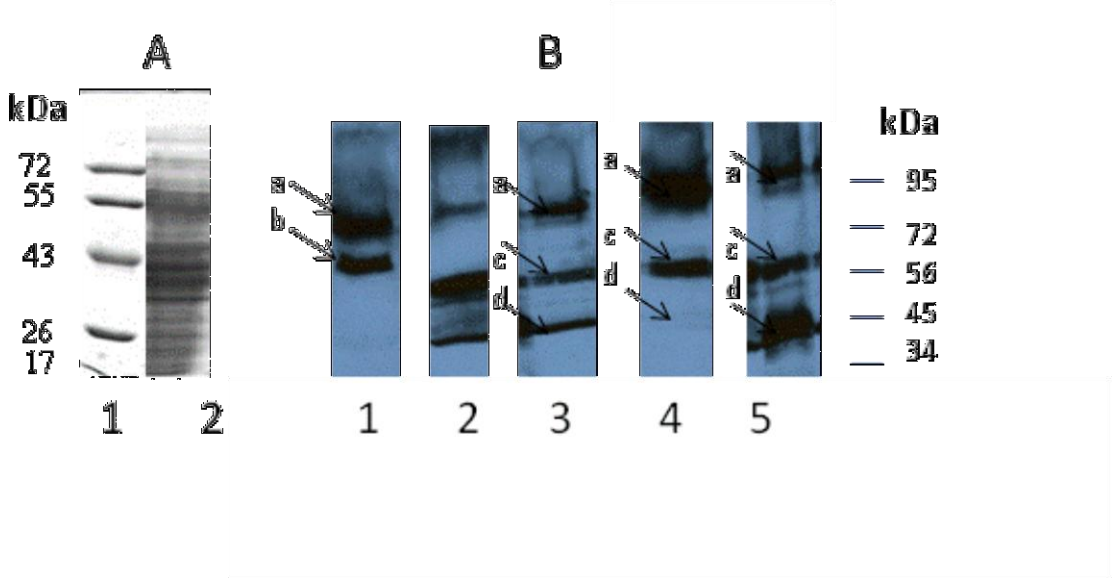

Table1

Table 1. Performance of fermentative H_2 production from different type of carbohydrates

Substrates	Fructose	Glucose	Sucrose G-(α 1,2)-F	Maltose G-(α 1,4)-G	Cellobiose G-(β 1,4)-G	Maltotriose G-(α 1,4)-G-(α 1,4)-G	Statistical significance[b]
P (L-H_2/L)[a]	2.31 ± 0.21	2.24 ± 0.15	2.16 ± 0.12	2.08 ± 0.08	2.00 ± 0.08	1.85 ± 0.16	*
R_m (L-H_2/L/d)[a]	0.91 ± 0.37	0.71 ± 0.19	0.92 ± 0.33	0.45 ± 0.03	0.41 ± 0.04	0.36 ± 0.08	*
λ (day)[a]	1.69 ± 0.32	1.66 ± 0.19	1.82 ± 0.29	1.64 ± 0.09	2.04 ± 0.09	2.02 ± 0.10	n.s.
R^2 (%)[a]	99.6 ± 0.4	99.5 ± 0.3	99.3 ± 0.6	98.7 ± 0.1	99.6 ± 0.2	99.3 ± 0.3	n.s.
H_2 yield (mol-H_2/mol-hexose)	1.84 ± 0.17	1.79 ± 0.12	1.67 ± 0.09	1.65 ± 0.06	1.56 ± 0.06	1.38 ± 0.12	**
R_c (mL/L)	55.9 ± 20.5	62.8 ± 15.4	30.5 ± 34.3	53.6 ± 35.1	84.4 ± 1.4	41.9 ± 21.5	n.s.
Max. H_2 content (%)	32.5 ± 1.1	34.2 ± 1.0	32.8 ± 0.7	32.7 ± 1.1	32.4 ± 0.6	31.0 ± 1.3	*
Final H_2 content (%)	22.8 ± 0.7	22.7 ± 1.3	27.6 ± 3.5	26.4 ± 2.7	22.9 ± 0.7	25.1 ± 0.8	*
Time when *P* was reached (days)	7.7 ± 1.2	7.7 ± 1.2	9.7 ± 0.6	9.7 ± 0.6	9.7 ± 0.6	9.7 ± 0.6	*
Final pH	4.0 ± 0.0	4.0 ± 0.0	4.0 ± 0.0	3.9 ± 0.0	4.0 ± 0.0	4.0 ± 0.0	n.s.

[a] Parameters derived from modified Gompertz equation
[b] Significant differences between substrate conditions are indicated by asterisks: **, $p<0.005$; *, $p<0.05$; n.s., not significant ($p>0.05$).

Table2

Table 2. Metabolite formation in fermentative mixed cultures from different carbohydrates

Substrates	Acetate (mmoles-Ac/mol-hexose)		Butyrate (mmoles-Bu/mol-hexose)		Caproate (mmoles-Ca/mol-hexose)		Ethanol (mmoles-Et/mol-hexose)		Lactate (mmoles-La/mol-hexose)		Bu/Ac ratio	
	P time	Final time	*P* time	Final time	*P* time	Final time	*P* time	Final time	*P* time	Final time	*P* time	Final time
Fructose	266.7	398.6	473.5	565.4	0.0	0.0	48.5	42.7	7.2	0.0	1.78	1.42
Glucose	245.8	218.0	449.9	310.5	0.0	0.0	45.8	0.0	6.5	0.0	1.83	1.42
Sucrose	370.2	271.3	540.5	333.8	0.0	20.6	59.7	56.2	6.6	0.0	1.46	1.23
Maltose	291.0	266.1	465.7	376.7	8.5	33.0	32.6	28.0	0.0	7.2	1.60	1.41
Cellobiose	309.3	279.7	454.7	327.1	10.8	43.2	78.6	41.4	18.8	0.0	1.47	1.17
Maltotriose	275.9	305.8	426.2	389.8	11.1	10.3	147.0	117.5	28.2	0.0	1.54	1.27

Table3

Table 3. Hydrogenase identification by metaproteomic analysis from Clostridium database. Proteins were identified by ion trap mass spectrometry from the band cut out from the SDS gel shown in Figure 4.

Band	N°	Protein name [a]	NCBI entry [b]	Coverage [c]	Peptides [d]	Mass [e]
a	1	Chaperonin GroEL	229261799	3.5	3	57.9
	2	Hypothetical protein	229261779	3.5	3	85.9
	3	FlaB	110164831	4.1	2	52.1
	4	**Hydrogenase Fe only**	220000195	4.5	4	62.5
	5	Chaperonin GroEL	18311271	2.4	2	65
	8	**Hydrogenase Fe only**	118444478 187779567	2.1	2	54.6
b	1	Conserved protein	28204348	7.6	*2*	48.5
	2	ATP dependent DNA ligase	219998149	13.4	*4*	36.8
	3	Putative Na antiporter	126698235	4.5	*2*	42.2
	4	**Ech hydrogenase subunit**	220930727	10.5	*4*	49.5
c	1	Transcriptor regulator Crp/Fnr	266620955	5.1	3	27.2
	2	SurE	255523330	4	4	27.4
	9	**Fe-Hydrogenase**	52854761	3.3	3	25.5
	12	**Fe-Hydrogenase**	255527517	2	1	46.8
d	53	NADH dehydrogenase	149905389	18.3	2	18.6

[a] Protein name is name as given in NCBI database.
[b] NCBI entry is the accession number.
[c] Coverage indicates protein sequence coverage by the matching peptides (in %).
[d] Peptides correspond to the number of different peptides matching to the protein sequence.
[e] Mass indicates the theoretical molecular mass in kDa of the identified protein calculated from the amino acid sequence without possible processing or modifications.